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14. ABSTRACT

Overexpression of EGFR is frequently linked to more aggressive tumor behavior, including increased proliferation, metastasis, and therapeutic resistance. Here, we identified a molecular linkage between IKKα and EGFR signaling in breast cancer cells. Inhibition of IKKs activity elevates EGFR tyrosine phosphorylation. In addition, IKKα forms a specific interaction with EGFR in Golgi apparatus and catalyzes EGFR S1026 phosphorylation. We found that EGFR S1026A possess a stronger tumorgenesis phenotype compare with wild type EGFR suggesting a negative regulation of IKKα in EGFR signaling. In agreement with an earlier finding where conditional ablation of IKKα in the mice keratinocytes elevates the autocrine loop of EGFR, our results further provide a potent role of IKKα kinase activity in preservation of EGFR activity.

15. SUBJECT TERMS
EGFR signaling, inflammatory kinase, IKKalpha, tumorgenesis, tumor suppressor

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1. INTRODUCTION:

A Triple-negative breast cancer (TNBC), which account for approximately 15-20% of breast cancers in the United States, lacks the expression of estrogen receptor (ER) and progesterone receptor (PR) as well as amplification of HER2/neu and is associated with poorer outcome compared with other breast cancer subtypes (*1-3*). TNBC also overlaps with the basal-like breast cancer, which is a subtype of breast cancer classified by genomic signatures identified in the molecular classification, although they are not same (*2, 4*). Unlike ER-positive, PR-positive, or HER2-overexpressing tumors, the lack of well-defined molecular targets and the heterogeneity of the disease pose a challenge for treating TNBC (*1, 3*).

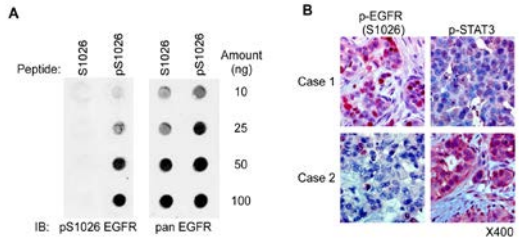
Aberrant activation and overexpression of the epidermal growth factor receptor (EGFR) contribute to aggressive tumor behavior and poor patient prognosis (*5*), and thus drugs that target EGFR are being used to treat many types of cancers. However, they are not as effective for breast cancer, suggesting that other mechanisms (*6, 7*) or biological functions of EGFR that have yet to be discovered may have important roles in breast cancer. Overexpression of EGFR has been frequently observed in TNBC and is associated with poor clinical outcome in TNBC patients (*4, 8*). These findings suggest that further understanding of the role of EGFR is critical for implementing successful anti-EGFR therapy in TNBC.

In this study, we found the inflammation regulator, IKKα, inhibits EGFR activity through a novel signaling pathway in breast cancer cells. IKKα binds to and phosphorylated EGFR at S1026. Inhibition of IKK activity led to hyperphosphorylation of EGFR Y845 and STAT3 705 suggesting a negative regulatory role of IKKα in breast cancer cells. Interestingly, low IKKα expression and high STAT3 activation was found in TNBC cells. These result suggest deregulation of IKKα contribute to the aggressive phenotype of TNBC cell. Altogether, our study provides novel mechanistic insight of IKKα mediated EGFR suppression in TNBC cells.

2. RESEARCH ACCOMPLISHMENTS BODY

Part I: Clinical relevance of pEGFR S1026 and p-STAT3

To recapitulate IKKα mediated EGFR S1026 phosphorylation, we purified and analyzed the phospho-EGFR S1026 antibody. As shown in the second year's progress report, IKKα induces a nice phosphorylation of EGFR using a p-EGFR S1026 antibody. Mutation of S1026 to analine (S1026A) abolishes IKKα mediated EGFR phosphorylation. The purified anti-phospho-S1026 EGFR antibody specifically recognized phospho-$EGFR^{WT}$ but not $EGFR^{S1026A}$ (Fig. 1A). To further determine the clinical relevance, we analyzed the correlation between phospho-S1026 EGFR and phosphor-Y705 STAT3 in human breast cancer tissues. A negative correlation was identified between phosho-S1026 EGFR and phosphor-Y705 STAT3 in human TNBC but not non-TNBC tissues (Fig. 1B and 1C). In addition, low level of phosho-S1026 and high level of phosphor-Y705 STAT3 correlated with a poor survival in TNBC patients cohorts (data not shown) suggesting their important roles in TNBC proliferation.



C

		Expression of p-EGFR				
		-/+	++	+++	Total	*P* value
p-STAT3	-/+	2 (1.3%)	8 (5.4%)	49 (32.9%)	59 (39.6%)	
	++	9 (6.0%)	5 (3.4%)	13 (8.7%)	27 (18.1%)	0.001*
	+++	45 (30.2%)	15 (10.1%)	3 (2.0%)	63 (42.3%)	
	Total	56 (37.6%)	28 (18.8%)	65 (43.6%)	149 (100.0%)	

Fig1. Clinical relevance of pEGFR S1026/STAT3 (A) Specificity of antibody against phospho-S1026 of EGFR was determined by dot blot assay. Nitrocellulose membrane was spotted with different amounts of phospho-S1026 peptides or unmodified peptides and probed with anti-phospho-S1026 antibody. **(B)** Representative IHC staining images of phospho-S1026 EGFR and phosphor-Y705 STAT3 in TNBC tissues. **(C)** Correlation between phospho-S1026 EGFR and phosphor-Y705 STAT3 in TNBC.

Part II: IKKα is downregulated in Triple Negative Breast Cancer (TNBC)

As we report in the last year's progress report, we examine gene expression profile of IKKα (NCBI gene ID: Chuk) using public data set (CCLE)(*9*) to identify the potential STAT3 downstream target that regulated by IKKα. We compared the expression profile of IKKα and 60 STAT3 downstream targets in breast cancer cells (*10*). Among then, 12 genes show negatively correlated with IKKα expression using CCLE. Nonsupervised hierarchical clustering analysis was performed based on Erbb2, ERα (ESR1), PR (PgR) profile. Strikingly, the gene list was able to distinguish basal-like from luminal type breast cancer cells with high accuracy (90% properly segregated) (Fig. 2A). To our surprise, IKKα expression was found to be downregulated in TNBC cells (Fig. 2B). We also analyzed an earlier identified STAT3 target regulated by IKKα through EGFR, CCL2. Consistently, CCL2 was found to be upregulated in TNBC cells (Fig. 2C).

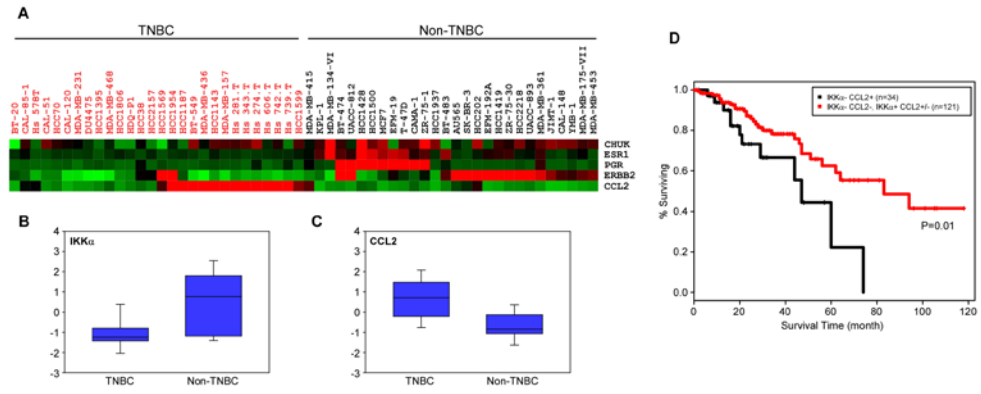


Figure 2. IKKα mRNA expression in CCLE 56 breast cancer cell lines. (**A**) Nonsupervised clustering of 55 breast cancer cell lines of EGFR and TNBC signature (*ERBB2*, *ESR1*, and *PGR*) genes. A subset of 4 genes showing distinct expression pattern in TNBC versus non-TNBC cell lines is shown in the heatmap. **(B)** Low expression of IKKα in TNBC cell lines. **(C)** Low expression of IKKα in TNBC cell lines. **(D)** Kaplan-Meier overall survival curves of IKKα/ in triple negative breast cancer patient data set.

We next asked if clinical distinct group of patient samples also shared the differential expression pattern of IKKα. First, we analyzed IKKα and CCL2 genes expression from Netherlands Cancer Institute (NKI) data set, *n*=295 (*11*). To do this, patients in the NKI cohort were first dichotomized according to their expression levels. As expected, low IKKα and high CCL2 of TNBC patients showed a significant difference in recurrence-free survival (RFS) compare to the rest of the status. Altogether, downregulation of IKKα and high expression of CCL2 is likely contributed to TNBC aggressive phenotype.

Part III: Downregulation of IKKα in Triple Negative Breast Cancer (TNBC)

Based on the data set mining, the newly identified mechanism is likely to be TNBC specific. To further validate the results from the database analysis, we analyzed the intrinsic IKKα/STAT3 activity and the alteration of mRNA expression in TNBC and non-TNBC cells. Consistently, TNBC cells show relatively low IKKα and high STAT3 activity using a reporter assay (Fig. 3A and 3B). Interestingly, the levels of mRNA expression CCL2 (Fig. 3C) but not STAT1 (Fig. 3D) is significantly increased in those TNBC cells. Together, these data indicate that IKKα mediated EGFR phosphorylation and inhibition is restricted in TNBC cells. Downregulation of IKKα in TNBC cells results in the activation of EGFR/Src/STAT3 loop and therefore activates STAT3 downstream target CCL2 (Fig. 3E).

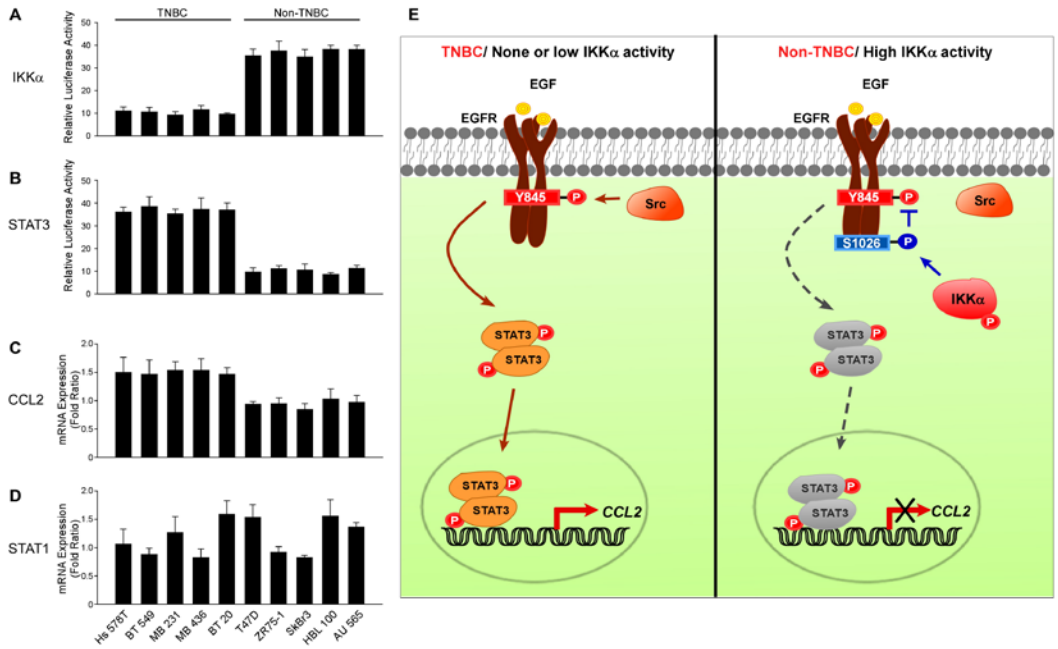


Figure 3. Validation of IKKα/EGFR/STAT3 in TNBC cells. (A) Low expression of IKKα in TNBC cells. TNBC and non-TNBC cells were transfected with the IKKa targeting-Luc. The luciferase activity was measured and normalized according to *Renilla* luciferase activity. **(B)** STAT3 is highly activated in TNBC cells. TNBC and non-TNBC cells were transfected with the lye6-Luc and measured by luciferase activity. The mRNA expression of CCL2 **(C)** and STAT1 **(D)**. **(E)** Proposed model of mechanism. Low expression of IKKα in TNBC cells results in EGFR/Src/STAT3 activation for their aggressive phenotype.

Part IV: inhibition of Src activity sensitize TNBC cells to TKI

Sorafenib (Nexavar, BAY43-9006), a multi-kinase inhibitor, inhibits Src mediated STAT3 activation in many type of cancer cells. However, sorafenib alone is not effective in breast cancer cells and has an unacceptable toxicity at high doses. Because gefitinib alone is not enough to kill breast cancer cells due to reported resistance in breast cancer cells, we tested the combination of sorafenib and gefitinib in to determine if sorafenib sensitizes TBNC cells to gefitinib. The combined treatment of gefitinib and sorafenib synergistically suppressed cellular proliferation in TNBC cells such as MDA-MB-231 and MDA-MB-468 (Fig. 4A and 4B) but not in non-TNBC cells such as MCF7 and T47D cells compared to either inhibitor alone (data not shown).

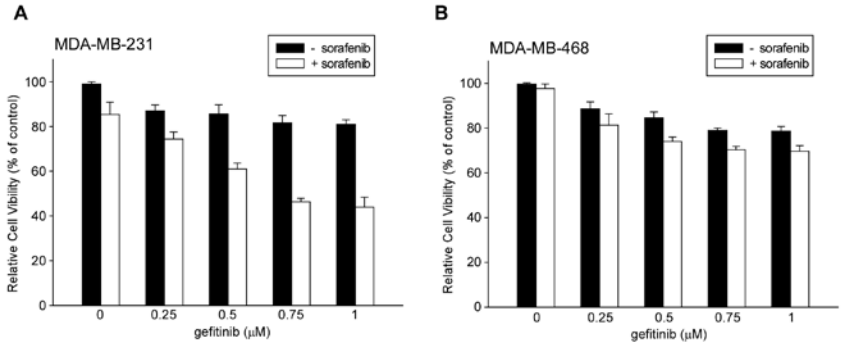


Figure 4. The combination treatment of EGFR TKI and Src inhibitor sorifinib in breast cancer cells. (A) MDA-MB-231 and **(B)** MDA-MB-468 cells were used for *in vitro* studies. Cell viability was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Part V: Tumor necrosis factor alpha-induces EMT required p65-mediated transcriptional upregulation of Twist1. Supported by DoD funding, the PI has accomplished another project unraveling tumor microenvironment-mediated breast cancer metastasis.

In the past three DoD funding years, we also identify that chronic exposure of pro-inflammatory cytokine, TNFα, in induces breast cancer cells EMT phenotypic changes and stemness, and subsequently identified Twist1 as a novel modulator of this regulation (*12*). Our results unravel the NFκB-mediated Twist1 upregulation as a novel therapeutic strategy for breast cancer treatment. In fact, this report has brought a lot of attention in the cancer research field. <u>One year non-self citation of this particular paper is now exceeding 30 times.</u>

Part VI: Phosphorylation of Twist1 by AKT1 Modulates Epithelial-Mesenchyme Transition in Breast Cancer Cells. Supported by DoD funding, the PI also serves as first author of another manuscript related to Triple Negative Breast Cancer (TNBC)

treatment (see attached abstract and figures).

Accumulating evidence from both cellular and genetic studies suggests AKT1/PKBα serves as a negative regulator of EMT during breast cancer metastasis. In this study, we found that AKT1 induced a phosphorylation-dependent ubiquitination and degradation of Twist1, engages the proteasome to Twist1-mediated EMT regulation. Our findings reveal a novel molecular concept by which non-specific inhibition of AKT may result in Twist1 stabilization to increase the metastatic potential in breast cancer cells. This manuscript is now under revised in Cancer Cell. The PI has used the past five month to address reviewer's questions and is now ready to response to Cancer Cell.

Part VII: EGFR Associates with and Primes GSK3β for its Inactivation and Mcl-1 upregulation. Supported by DoD funding, the PI also serves as first author of another manuscript related to Triple Negative Breast Cancer (TNBC) treatment (see attached abstract).

In studies with endogenous GSK3β association complex, we identified EGFR as a novel GSK3β-interacting protein, which phosphorylates GSK3β and inhibits GSK3β activation. We revealed that GSK3β's activity is stringently modulated by a previously unknown and reversible modification, ubiquitination through a distinct TRAF6 binding motif of GSK3β. The essence of PE motif for enhancing GSK3β activity suggested TRAF6-mediated K63 ubiquitinaiton is involved. Furthermore, TRAF6 activates GSK3β activity, thereby affecting GSK3β dependent apoptosis. Altogether, we demonstrate EGFR associates with and phosphorylates GSK3β, which primed inactivation of GSK3β by inhibiting TRAF6-mediated ubiquitination, resulting in Mcl-1 upregulation.

3. CONCLUSION

EGFR, as an essential growth and survival factor, plays an important role in many cancer types. The modification patterns of EGFR are critical for its function and the understanding of these EGFR modifications could help us design the optimal therapeutic strategies for targeting various EGFR-associated cancers and/or non-cancerous diseases. We herein identified a novel posttranslational modification of EGFR which plays an indispensable role in regulation of EGFR signaling pathways. We found that IKKα is responsible for EGFR S1026 serine phosphorylation. S1026 phosphorylation of EGFR negatively impacts its synergic interaction with Src. Similar to other serine/theronine phosphorylation on the EGFR, phosphorylation by IKKα downregulates EGFR signaling and thereby diminishes cell growth and tumorigenesis.

The third year research is focusing on the pathological identification of the novel signaling in breast cancer cells. Using CCLE dataset analysis, we first identified a negative correlation of IKKα and TNBC cells. We went on to identify CCL2 as a novel downstream target of IKKα/EGFR/STAT3 signaling axis. Biochemical analysis consolidates the involvement of CCL2 in TNBC cells. We also analysis two patient data set and found out the poor prognosis of low IKKα and high CCL2. This result provides the first evidence showing the inhibitory nature of IKKα in both cell based study and patient samples.

In conclusion, the proposed experiments by the PI have accomplished. In the past three years, the PI identified the novel phosphorylation on EGFR by IKKα at S1026. Functional analysis indicate IKKα trigger a negative regulation by interfering EGFR/Src interaction. Database analysis further suggests the inhibitory nature of IKKα in TNBC cells and therefore possesses high level of STAT3 activity and CCL2 expression. In addition, we also breed MMTV-IKKα$^{-/-}$/EGFR mice in FVB background. Indeed, our preliminary data indicate that mice lack of IKKα accelerate hyperplastic lesion (please second year report). As the animal facility at MD Anderson cancer center has recently undergo rederivation process, we have request no cost extension to collect more MMTV-IKKα$^{-/-}$/EGFR mice to reach statistical significance.

4. FUTURE WORKS:

The MMTV-hEGFR transgenic mice developed mammary epithelial hyperplasias, hypertrophy, or slight dysplasias in about 55% of mammary glands of animals examined. Since the inhibition of IKKα results in hyperactivation of EGFR to provide a survival advantage for cancer cells, we plan to create conditional knock out of IKKα in mammary gland and cross with EGFR overexpression mice to measure tumor onset. The age of the mouse in which mammary tumor is first palpable will be recorded and tumor size will be measured. Although our preliminary data indicate that mice lack of IKKα accelerate hyperplastic lesion, deletion of IKKα enhances EGFR mediated tumorigenesis remains unknown. We are now breading more IKKα$^{-/-}$/EGFR mice to reach statistical significance.

5. NO COST EXTENSION:

To ensure adequate completion of the originally approved project, the PI requested no cost extension of grant number **W81XWH-10-1-0598** for a period of twelve months, commencing (09/14/2013) and ending on (09/14/2014).

As the animal facility of MDACC underwent conversion to specific-pathogen-free (SPF) status similar to the CSPF status we had in the basement of the BSRB. The institution required that all animals in this area that need to be maintained undergo a rederivation process to clean up the strain. This work has done by our Genetically Engineered Mouse Facility (GEMF) and the whole rederivation process has taken over twelve month to finish. Owing to this unexpected situation, the PI needs to collect the rest of the data of transgenic mice experiments. Once the results are collected, the data will be evaluated for publication. Therefore, an extended research period is required.

6. KEY RESEARCH ACCOMPLISHMENTS: 2012-2013

a) Complete biological function of EGFR S1026A *in vivo*. New data is not included in the third year report.

b) Identification of IKKα as negative regulator in Triple Negative Breast Cancer cells.

c) Characterization of phospho-EGFR S1026 antibody. EGFR S1026 phsophorylation is negative correlated with p-STAT3 in TNBC cells

d) Identify CCL2 as IKKα/EGFR/STAT3 downstream target. CCL2 is highly expressed in the TNBC cells.

e) Sorafenib and Gefitinib show combinatory effect in treating TNBC cells.

f) Two papers related to Triple Negative Breast Cancer (PI is the first author) have either been revised or ready for submission.

7. REPORTABLE OUTCOMES 2012-2013

Li CW, Xia W, Huo L, Lim SO, Wu Y, Hsu JL, Chao CH, Yamaguchi H, Yang NK, Ding Q, Wang Y, Lai YJ, Labaff AM, Wu TJ, Lin BR, Yang MH, Hortobagyi GN, Hung MC. (2012) *Epithelial-Mesenchymal Transition Induced by TNF-α Requires NF-κB-Mediated Transcriptional Upregulation of Twist1*.**Cancer Research** 72(5): 1290-1300

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Li CW, Ding Q, Xia W, Lai CC, Wang Y, Huo L, Li HJ, Lamothe B, Campos AD, Hur L, Lai YJ, Darnay BG and Hung MC. (2013*) The role of IKKα in EGFR signaling regulation*. *In submission*

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9. APPENDICES:

A. Abstract and Figures of recent manuscript revised in Cancer Cell

AKT1-mediated Inhibition of Breast Cancer Epithelial-Mesenchyme Transition Requires Phosphorylation-dependent Twist1 Degradation

Running Title: AKT1 induces β-TrCP-mediated Twist1 degradation

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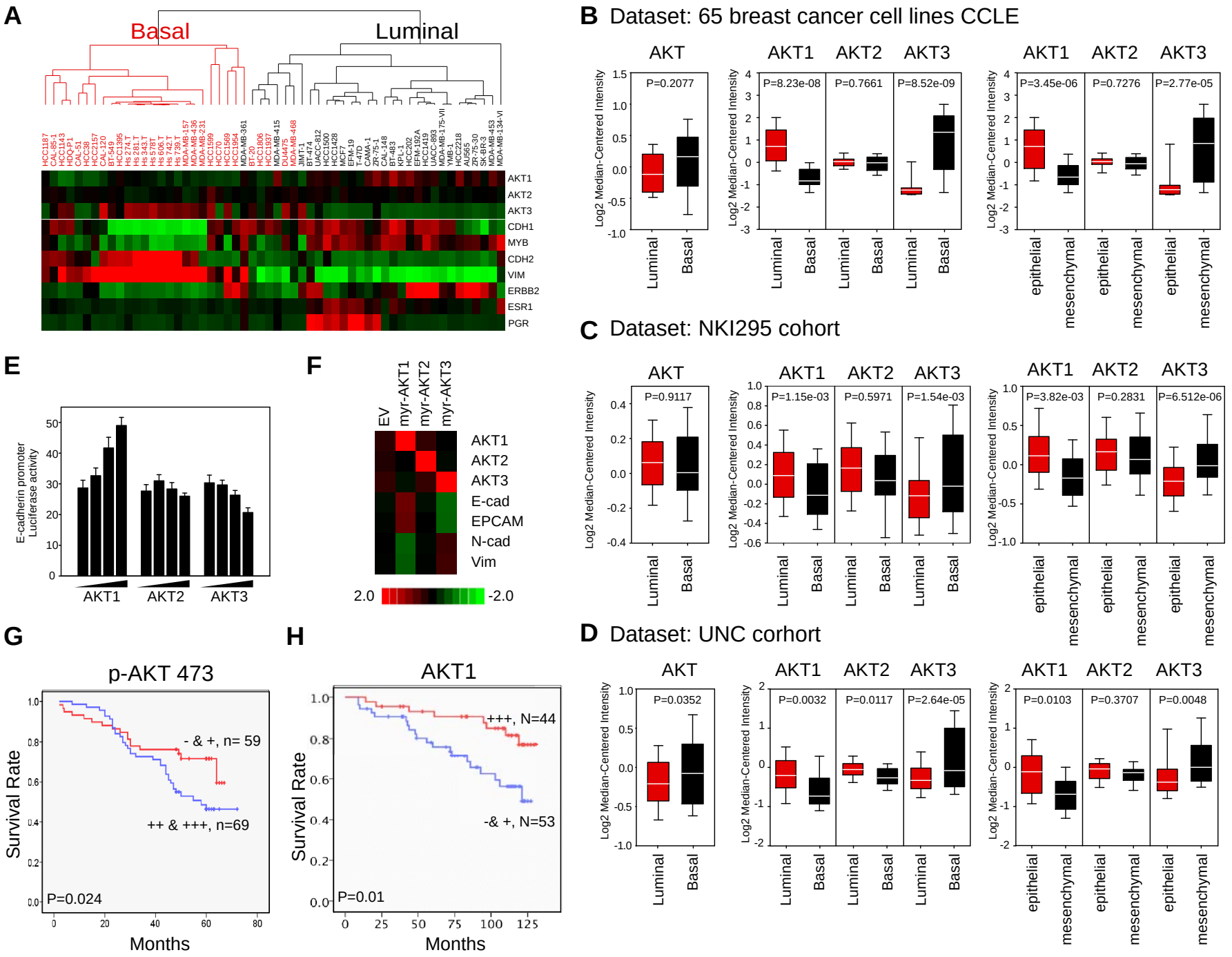
SUMMARY

Epithelial-to-mesenchyme transition (EMT) is an essential physiological process that promotes cancer cell migration, invasion, and metastasis. Several lines of evidence from both cellular and genetic studies suggest AKT1/PKBα serves as a negative regulator of EMT and breast cancer metastasis while AKT2 and AKT3 generally act as oncogenes to promote tumorigenesis. However, the underlying mechanism by which AKT1 suppresses EMT remains poorly defined. Here, we demonstrate that Twist1 phosphorylated by AKT1 is required for β-TrCP-mediated Twist1 ubiquitination and degradation. The clinically used AKT inhibitor MK-2206, which possesses higher specificity toward AKT1, stabilizes Twist1 and enhances EMT in breast cancer cells. This adverse effect can be overcome by combinatory therapy of MK-2206 and resveratrol to induce β-TrCP mediated Twist1 degradation.

Figure 1


A
Basal
Luminal
AKT1
AKT2
AKT3
CDH1
MYB
CDH2
VIM
ERBB2
ESR1
PGR
B Dataset: 65 breast cancer cell lines CCLE
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AKT1 AKT2 AKT3
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P=0.7276
P=2.77e-05
Luminal
Basal
epithelial
mesenchymal
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P=2.64e-05
P=0.0103
P=0.3707
P=0.0048
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E-cadherin promoter Luciferase activity
AKT1
AKT2
AKT3
F
EV
myr-AKT1
myr-AKT2
myr-AKT3
AKT1
AKT2
AKT3
E-cad
EPCAM
N-cad
Vim
2.0
-2.0
G
p-AKT 473
Survival Rate
- & +, n= 59
++ & +++, n=69
P=0.024
Months
H
AKT1
+++, N=44
-& +, N=53
P=0.01
Months

Figure 2

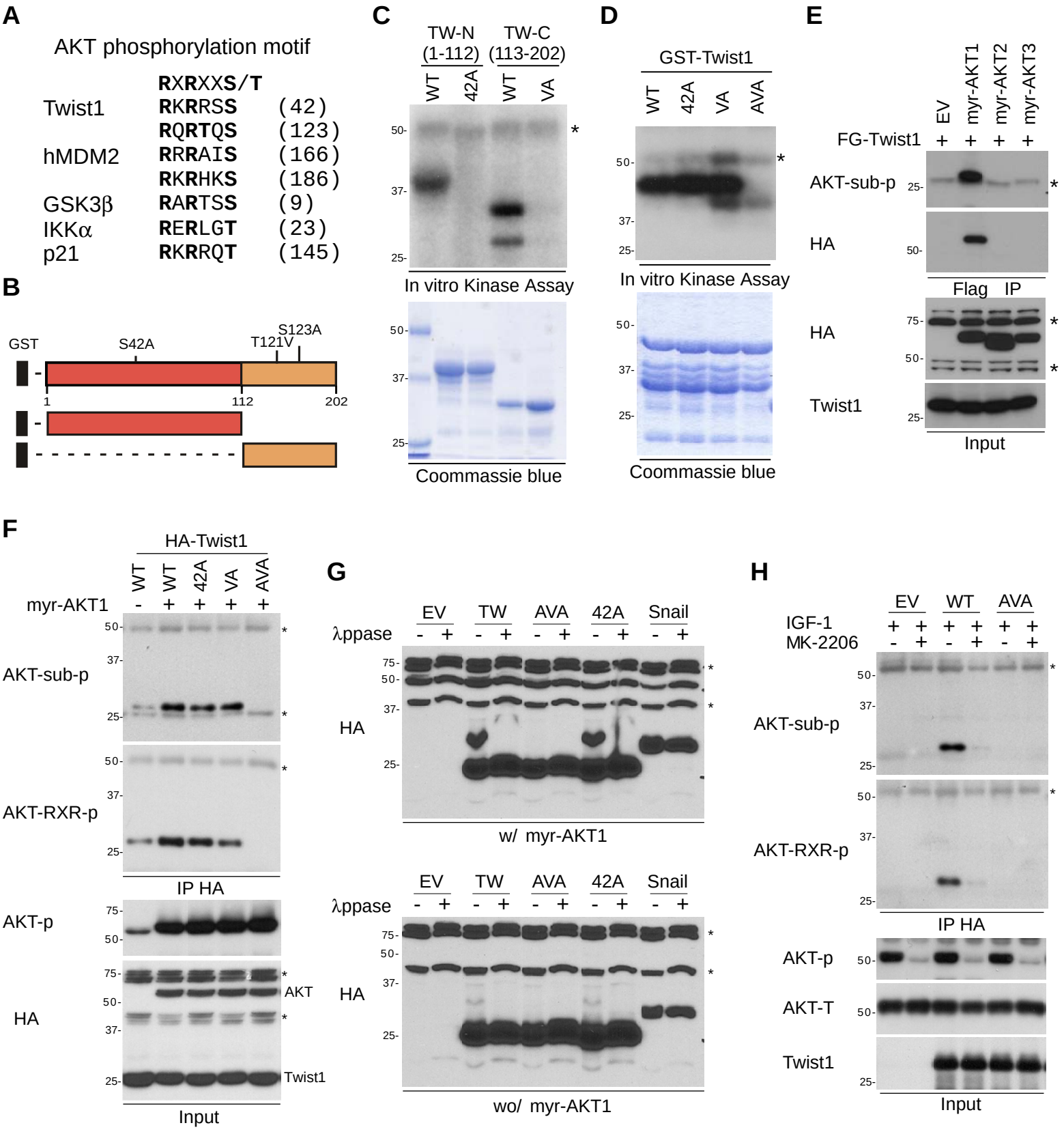

A
AKT phosphorylation motif
RXRXXS/T
Twist1 RKRRSS (42)
RQRTQS (123)
hMDM2 RRRAIS (166)
RKRHKS (186)
GSK3β RARTSS (9)
IKKα RERLGT (23)
p21 RKRRQT (145)
B
GST
S42A
S123A
T121V
1
112
202
C
TW-N (1-112)
TW-C (113-202)
WT
42A
WT
VA
In vitro Kinase Assay
Coommassie blue
D
GST-Twist1
WT
42A
VA
AVA
In vitro Kinase Assay
Coommassie blue
E
EV
myr-AKT1
myr-AKT2
myr-AKT3
FG-Twist1
AKT-sub-p
HA
Flag IP
HA
Twist1
Input
F
HA-Twist1
WT
WT
42A
VA
AVA
myr-AKT1
AKT-sub-p
AKT-RXR-p
IP HA
AKT-p
HA
AKT
Twist1
Input
G
EV
TW
AVA
42A
Snail
λppase
HA
w/ myr-AKT1
wo/ myr-AKT1
H
EV
WT
AVA
IGF-1
MK-2206
AKT-sub-p
AKT-RXR-p
IP HA
AKT-p
AKT-T
Twist1
Input

Figure 3

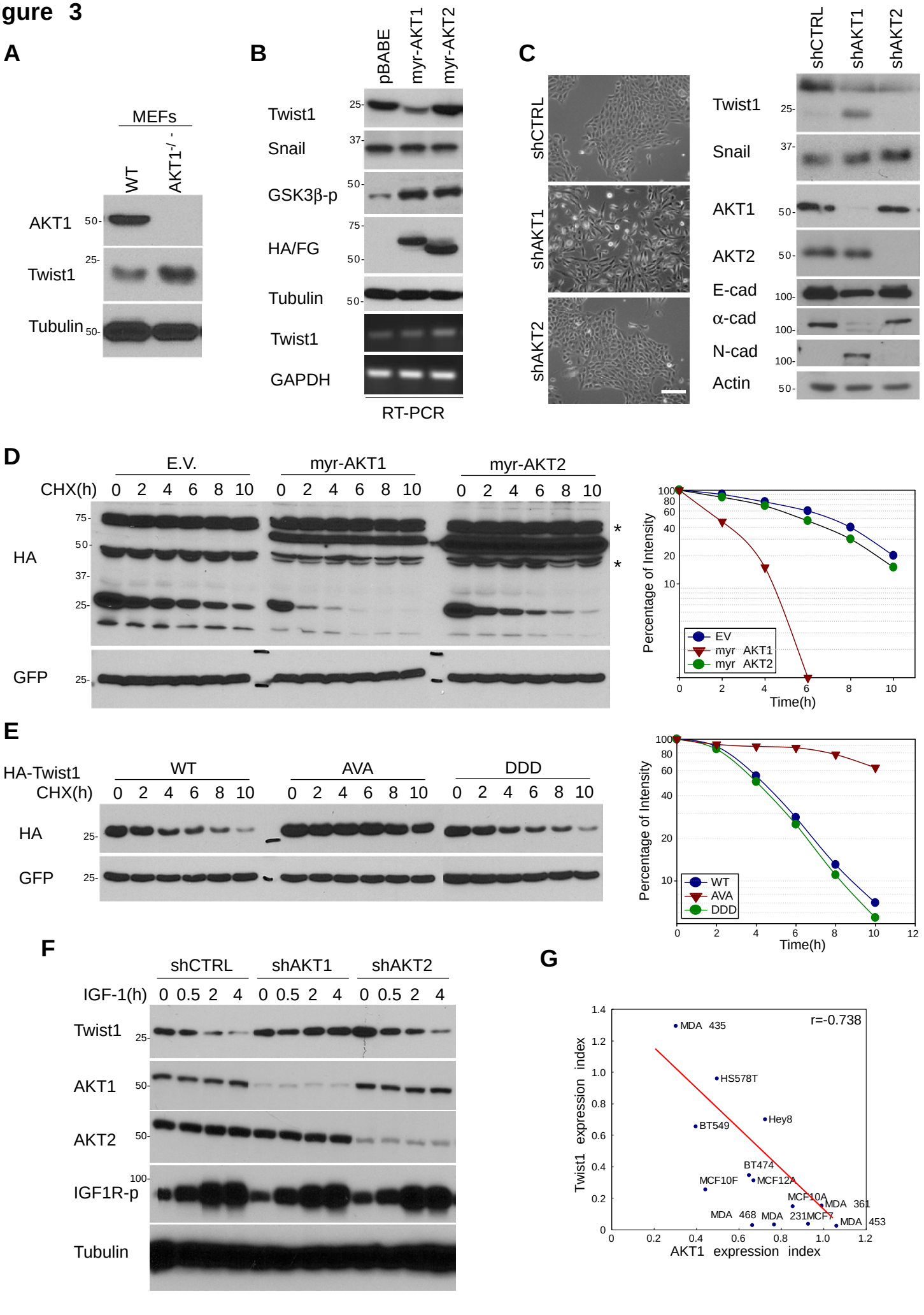

A
MEFs
WT
AKT1-/-
AKT1
Twist1
Tubulin
B
pBABE
myr-AKT1
myr-AKT2
Twist1
Snail
GSK3β-p
HA/FG
Tubulin
Twist1
GAPDH
RT-PCR
C
shCTRL
shAKT1
shAKT2
Twist1
Snail
AKT1
AKT2
E-cad
α-cad
N-cad
Actin
D
E.V.
myr-AKT1
myr-AKT2
CHX(h) 0 2 4 6 8 10
HA
GFP
Percentage of Intensity
Time(h)
EV
myr AKT1
myr AKT2
E
HA-Twist1
WT
AVA
DDD
CHX(h) 0 2 4 6 8 10
HA
GFP
Percentage of Intensity
Time(h)
WT
AVA
DDD
F
shCTRL
shAKT1
shAKT2
IGF-1(h) 0 0.5 2 4
Twist1
AKT1
AKT2
IGF1R-p
Tubulin
G
r=-0.738
MDA 435
HS578T
Hey8
BT549
BT474
MCF10F
MCF12A
MCF10A
MDA 361
MDA 468
MDA 231
MCF7
MDA 453
Twist1 expression index
AKT1 expression index

Figure 4

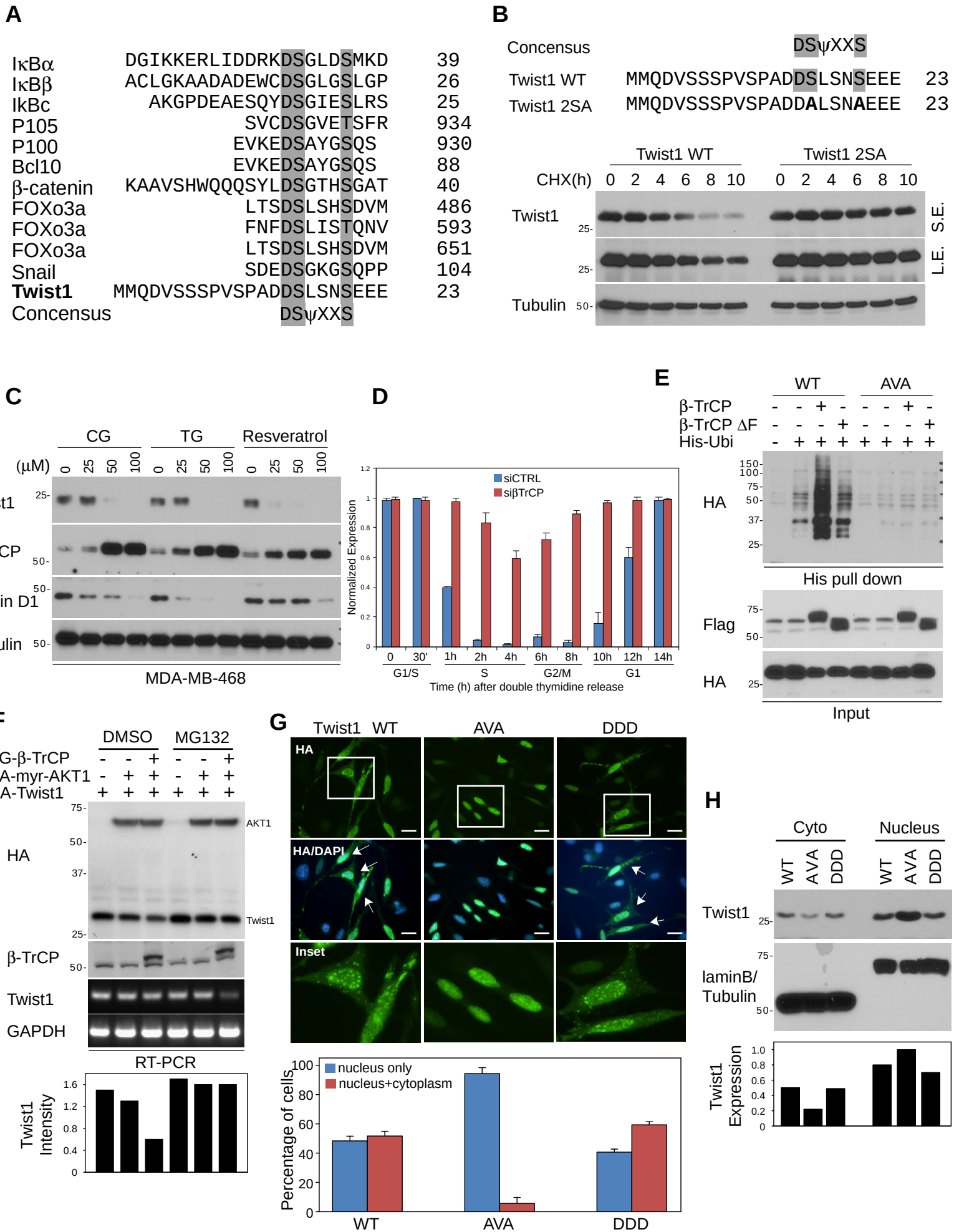

A
IκBα DGIKKERLIDDRKDSGLDSMKD 39
IκBβ ACLGKAADADEWCDSGLGSLGP 26
IkBc AKGPDEAESQYDSGIESLRS 25
P105 SVCDSGVETSFR 934
P100 EVKEDSAYGSQS 930
Bcl10 EVKEDSAYGSQS 88
β-catenin KAAVSHWQQQSYLDSGTHSGAT 40
FOXo3a LTSDSLSHSDVM 486
FOXo3a FNFDSLISTQNV 593
FOXo3a LTSDSLSHSDVM 651
Snail SDEDSGKGSQPP 104
Twist1 MMQDVSSSPVSPADDSLSNSEEE 23
Concensus DSψXXS
B
Concensus DSψXXS
Twist1 WT MMQDVSSSPVSPADDSLSNSEEE 23
Twist1 2SA MMQDVSSSPVSPADDALSNAEEE 23
Twist1 WT
Twist1 2SA
CHX(h) 0 2 4 6 8 10 0 2 4 6 8 10
Twist1
Tubulin
S.E.
L.E.
C
CG
TG
Resveratrol
(μM) 0 25 50 100 0 25 50 100 0 25 50 100
Twist1
β-TrCP
Cyclin D1
Tubulin
MDA-MB-468
D
siCTRL
siβTrCP
Normalized Expression
0 30' 1h 2h 4h 6h 8h 10h 12h 14h
G1/S S G2/M G1
Time (h) after double thymidine release
E
WT
AVA
β-TrCP
β-TrCP ΔF
His-Ubi
HA
His pull down
Flag
HA
Input
F
DMSO
MG132
FG-β-TrCP
HA-myr-AKT1
HA-Twist1
HA
AKT1
Twist1
β-TrCP
Twist1
GAPDH
RT-PCR
Twist1 Intensity
G
Twist1 WT
AVA
DDD
HA
HA/DAPI
Inset
nucleus only
nucleus+cytoplasm
Percentage of cells
WT
AVA
DDD
H
Cyto
Nucleus
WT AVA DDD WT AVA DDD
Twist1
laminB/ Tubulin
Twist1 Expression

Figure 5

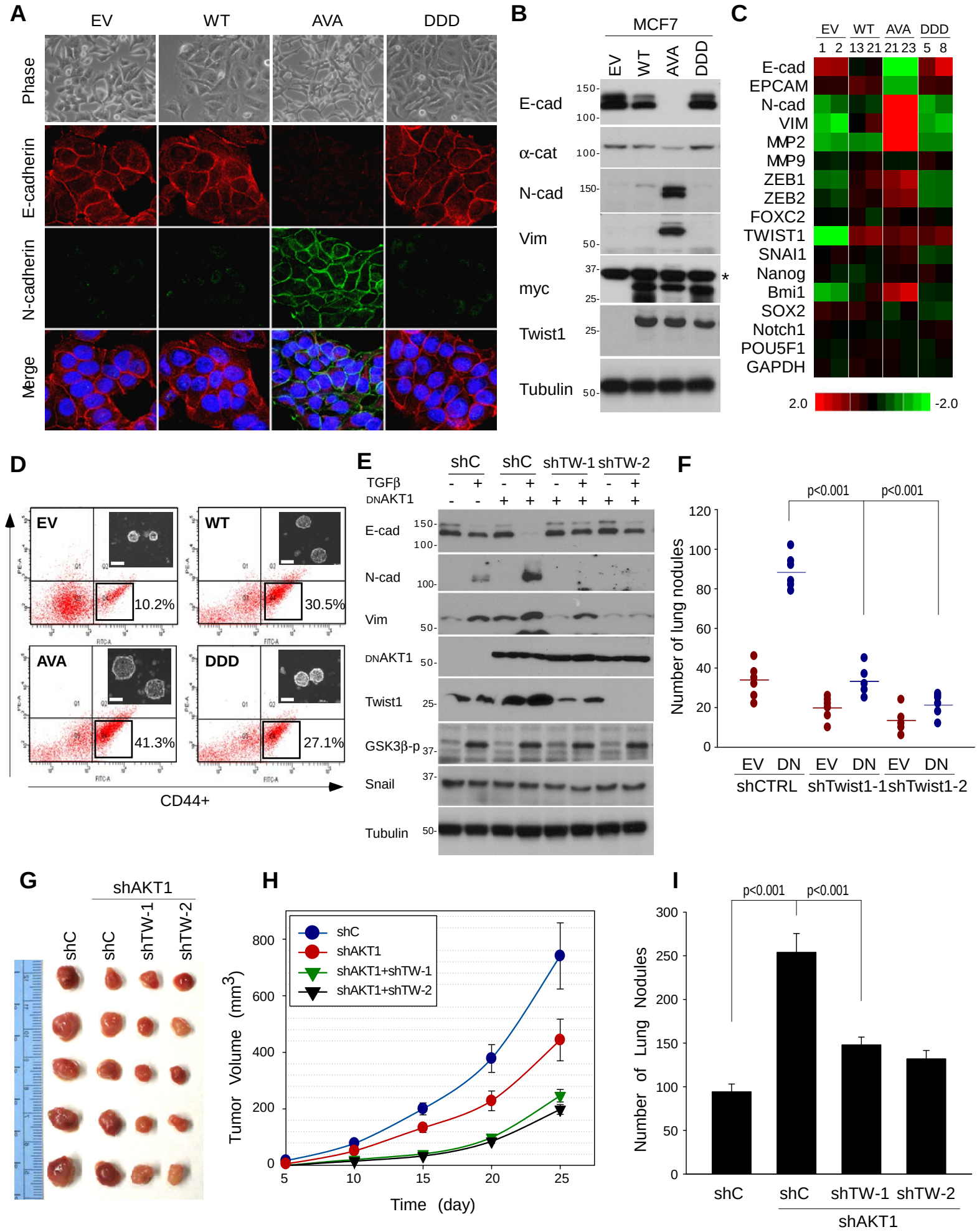

A
EV
WT
AVA
DDD
Phase
E-cadherin
N-cadherin
Merge
B
MCF7
EV
WT
AVA
DDD
E-cad
α-cat
N-cad
Vim
myc
Twist1
Tubulin
150-
100-
50-
37-
25-
*
C
EV WT AVA DDD
1 2 13 21 21 23 5 8
E-cad
EPCAM
N-cad
VIM
MMP2
MMP9
ZEB1
ZEB2
FOXC2
TWIST1
SNAI1
Nanog
Bmi1
SOX2
Notch1
POU5F1
GAPDH
2.0
-2.0
D
EV
WT
AVA
DDD
10.2%
30.5%
41.3%
27.1%
CD24-
CD44+
E
shC
shC
shTW-1
shTW-2
TGFβ
DNAKT1
E-cad
N-cad
Vim
DNAKT1
Twist1
GSK3β-p
Snail
Tubulin
150-
100-
50-
37-
25-
F
p<0.001
p<0.001
Number of lung nodules
120
100
80
60
40
20
0
EV DN EV DN EV DN
shCTRL shTwist1-1 shTwist1-2
G
shAKT1
shC
shC
shTW-1
shTW-2
H
shC
shAKT1
shAKT1+shTW-1
shAKT1+shTW-2
Tumor Volume (mm3)
800
600
400
200
0
5
10
15
20
25
Time (day)
I
p<0.001
p<0.001
Number of Lung Nodules
300
250
200
150
100
50
0
shC
shC
shTW-1
shTW-2
shAKT1

Figure 6

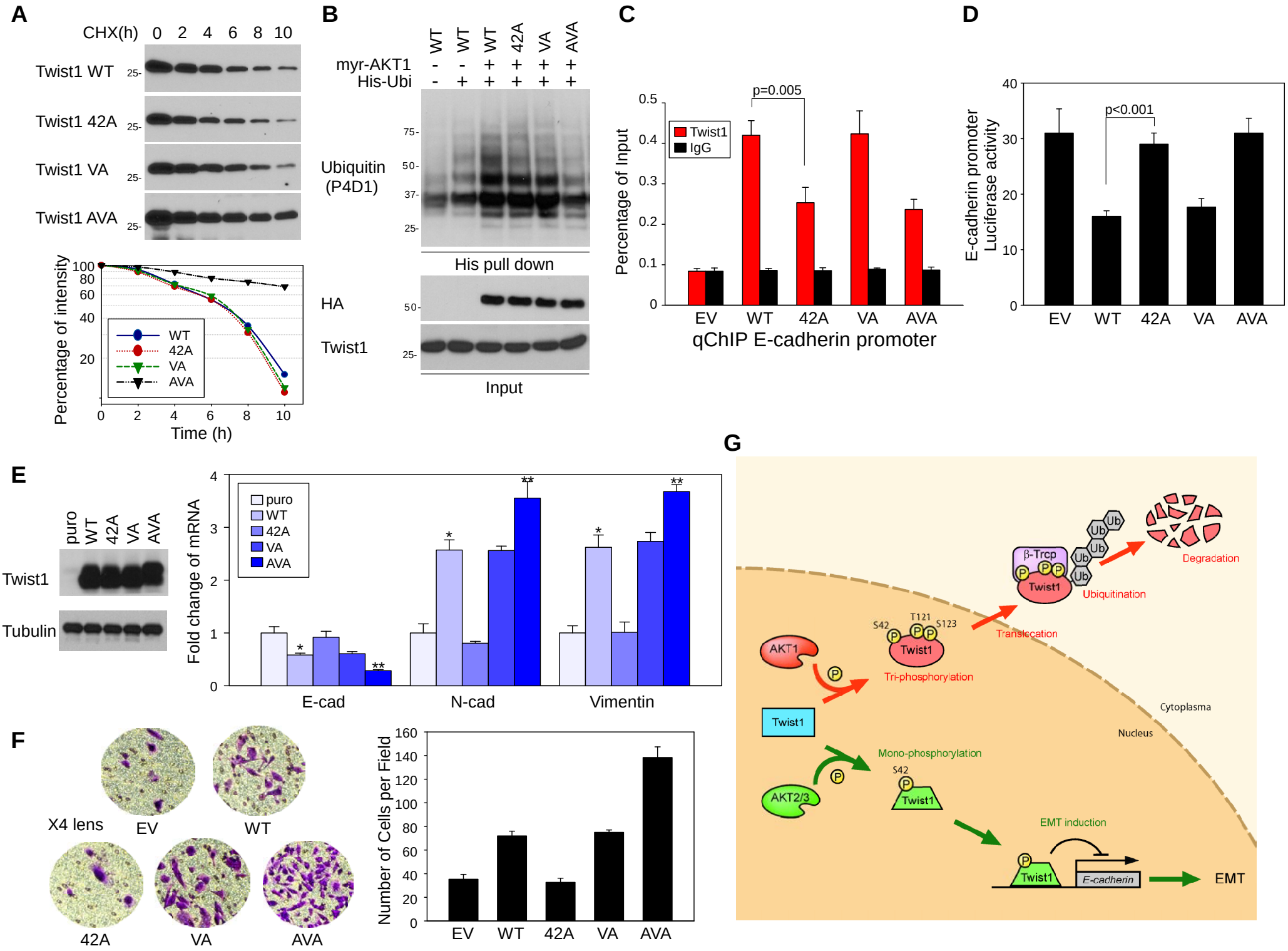
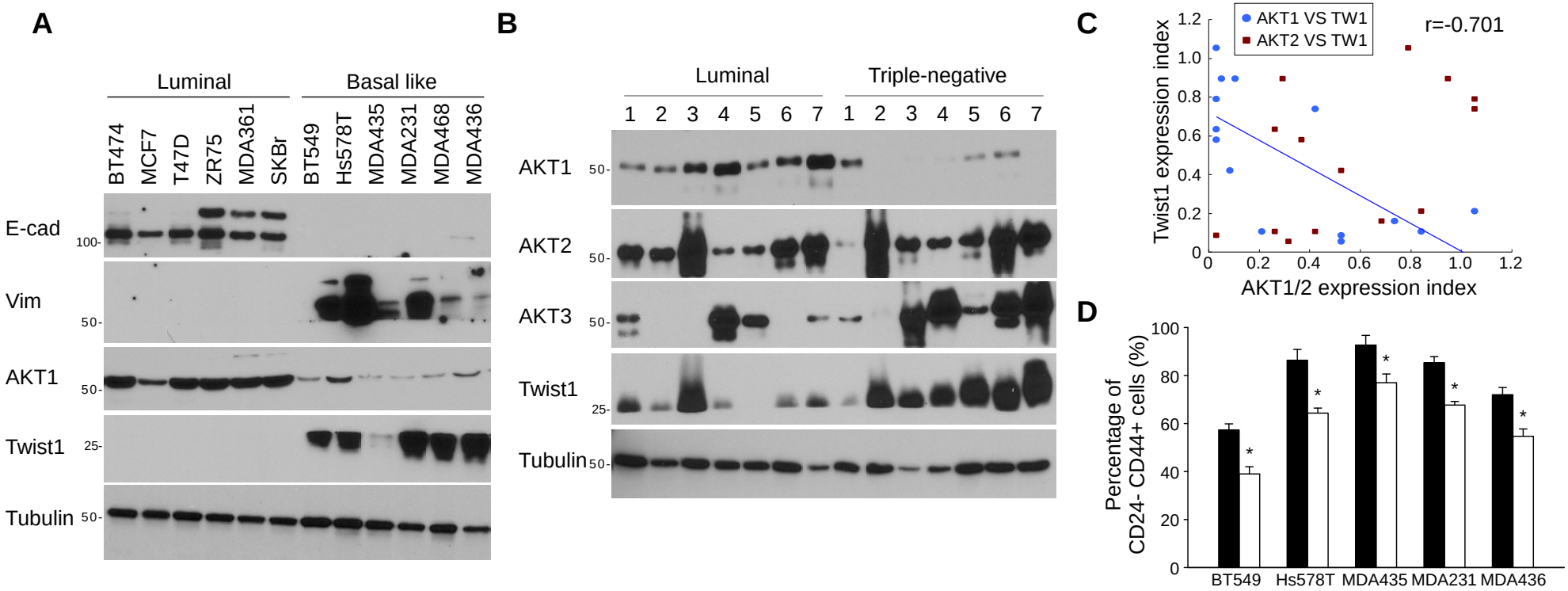
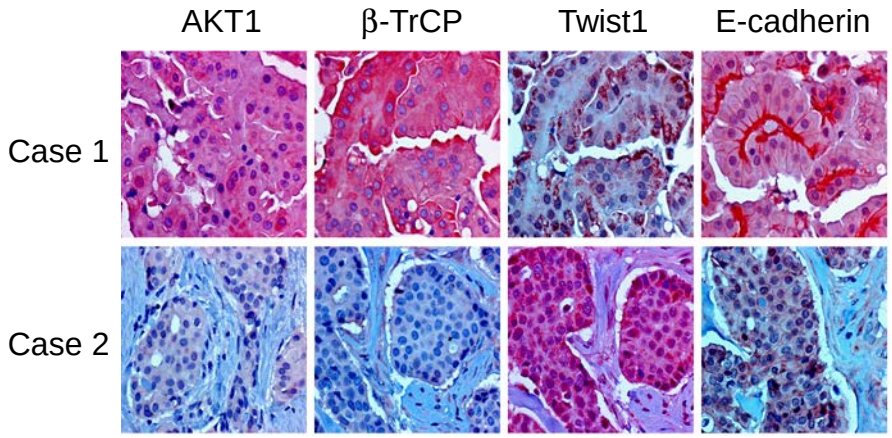

A
CHX(h) 0 2 4 6 8 10
Twist1 WT
Twist1 42A
Twist1 VA
Twist1 AVA
25-
Percentage of intensity
100
80
60
40
20
WT
42A
VA
AVA
0 2 4 6 8 10
Time (h)
B
WT WT WT 42A VA AVA
myr-AKT1 - - + + + +
His-Ubi - + + + + +
Ubiquitin (P4D1)
75-
50-
37-
25-
His pull down
HA
Twist1
50-
25-
Input
C
Percentage of Input
0.5
0.4
0.3
0.2
0.1
0
Twist1
IgG
p=0.005
EV WT 42A VA AVA
qChIP E-cadherin promoter
D
E-cadherin promoter Luciferase activity
40
30
20
10
0
p<0.001
EV WT 42A VA AVA
E
puro WT 42A VA AVA
Twist1
Tubulin
Fold change of mRNA
4
3
2
1
0
puro
WT
42A
VA
AVA
*
**
E-cad N-cad Vimentin
F
X4 lens
EV
WT
42A
VA
AVA
Number of Cells per Field
160
140
120
100
80
60
40
20
0
EV WT 42A VA AVA
G
β-Trcp
Ub
Twist1
Degradation
Ubiquitination
Translocation
S42 T121 S123
Tri-phosphorylation
AKT1
P
Twist1
Cytoplasma
Nucleus
Mono-phosphorylation
S42
AKT2/3
EMT induction
E-cadherin
EMT

Figure 7

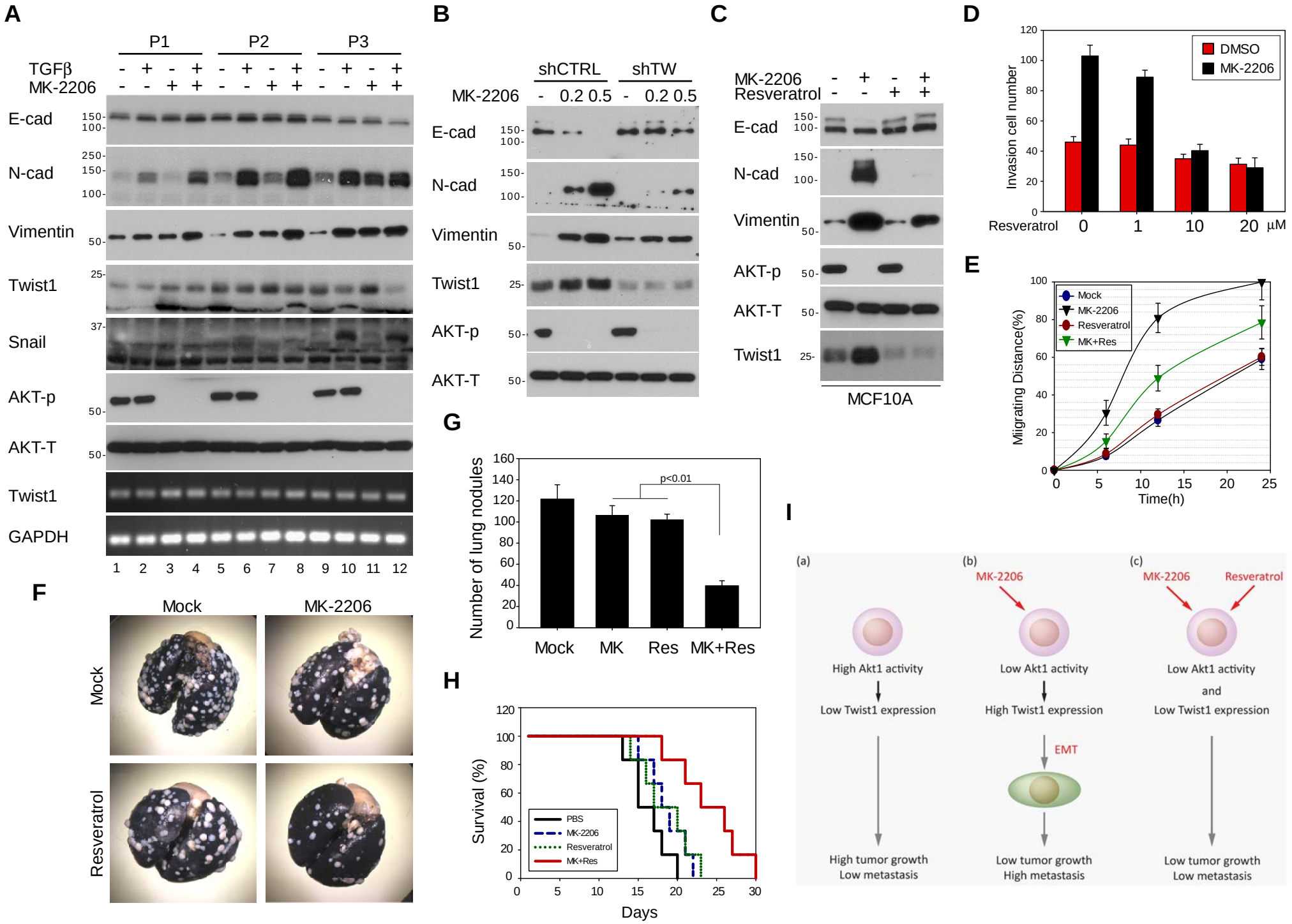




F

		AKT1 Expression				
		- / +	++	+++	Total	p value
β-TrCP	-/+	15(14.4%)	8(7.7%)	1(1.0%)	24(23.1 %	
	++	7(6.7%)	17(16.3%)	5(4.8%)	29(27.9)%	
	+++	1(1.0%)	8(7.7%)	42(40.4%)	51(49.0)%	
Total		23(22.1 %)	33(31.7%)	48(46.2%)	104 (100)%)	P= 0.0001
Twist1	-/+	6(5.8%)	9(8.7%)	13(12.6%)	28(27.2%)	
	++	21(20. 4%)	3(2.9%)	17(16.5%)	41(39.8%)	
	+++	18(17.5%)	7(6.8%)	9(8.7%)	34(33.0%)	
Total		45(43.7%)	19(18.4%)	39(37.9%)	103(100%)	P=0.018
E-Cad	-/+	12(14.5%)	7(8.4%)	3(3.6%)	22(26.5 %	
	++	5(6.0%)	9(10.8%)	14(16.9%)	28 (33.7)%	
	+++	4(4.8%)	10(12%)	19(22.9%)	33 (39.8)%	
Total		21(25.3%)	26(31.3 %)	36(43.4 %)	83(100%)	P= 0.002

Figure 8


A
P1
P2
P3
TGFβ
MK-2206
E-cad
N-cad
Vimentin
Twist1
Snail
AKT-p
AKT-T
Twist1
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12
B
shCTRL
shTW
MK-2206 - 0.2 0.5 - 0.2 0.5
E-cad
N-cad
Vimentin
Twist1
AKT-p
AKT-T
C
MK-2206
Resveratrol
E-cad
N-cad
Vimentin
AKT-p
AKT-T
Twist1
MCF10A
D
DMSO
MK-2206
Invasion cell number
Resveratrol 0 1 10 20 μM
E
Mock
MK-2206
Resveratrol
MK+Res
Migrating Distance(%)
Time(h)
F
Mock
MK-2206
Mock
Resveratrol
G
Number of lung nodules
p<0.01
Mock MK Res MK+Res
H
Survival (%)
PBS
MK-2206
Resveratrol
MK+Res
Days
I
(a)
(b)
(c)
MK-2206
Resveratrol
High Akt1 activity
Low Twist1 expression
High tumor growth
Low metastasis
Low Akt1 activity
High Twist1 expression
EMT
Low tumor growth
High metastasis
Low Akt1 activity
and
Low Twist1 expression
Low tumor growth
Low metastasis